# CXLIV. ENZYME FORMATION AND POLYSACCHARIDE SYNTHESIS BY BACTERIA. II.

By ALBERT CARRUTHERS[1] and EVELYN ASHLEY COOPER.[2]

*From the Chemical Department, University of Birmingham.*

(*Received April 11th, 1936.*)

An account was given in a previous communication [Cooper and Preston, 1935] of the study of the biochemical conditions influencing the formation and activity of diastase and invertase in bacteria, and also of the synthesising enzyme associated with the formation of polysaccharides of the "laevan" type by bacterial action. It was suggested in that paper that the investigations would be of wider physiological interest if they could be extended to the formation of other polysaccharides such as dextrans, galactans, pentosans, in addition to laevans. Experiments in this direction were commenced with numerous micro-organisms, but in the case of the aerobic sporing bacilli and the plant-pathogenic bacteria their synthetic capacity was limited to "laevan formation".

Further investigations with other micro-organisms have now been carried out and are described in the present paper.

The general experimental methods and basal culture media employed were the same as those used in the previous work, the principal culture medium being a solution of

| | % |
|---|---|
| Peptone | 0·1 |
| $Na_2HPO_4, 12H_2O$ | 0·2 |
| KCl | 0·5 |
| Carbohydrate | 10 |

## Section I.

The micro-organisms tabulated in Table I were cultivated in 10 or 20 ml. portions of the foregoing medium containing 10% of various carbohydrates. The cultures were incubated at the optimum temperature of the particular organism for 7–10 days, and evidence of polysaccharide synthesis was looked for by filtering the cultures and allowing the filtrate to run into three times the volume of alcohol with mechanical stirring. The polysaccharide was thrown out as a white precipitate and purified by redissolving in water and precipitating twice with alcohol, grinding with alcohol and then ether and finally drying in a vacuum desiccator.

Table I.

| Micro-organism | Polysaccharide formation |
|---|---|
| *Bacillus lactis* | "Fructosan" from sucrose only |
| *Bact. fluorescens liquefaciens* | Nil |
| *Bact. acidi lactici* | Nil |
| *Bact. hyacinthi* | Trace from sucrose |
| *Chromobacter viscosum* | Trace from sucrose and galactose |
| *Micrococcus lactis viscosus* | Nil |

[1] On leave of absence from the Peiping Union Medical College, Peiping, China.

[2] Leverhulme Research Fellow.

*Bacillus lactis*, occurring in milk, readily formed a polysaccharide from sucrose. This was laevorotatory and was readily hydrolysed by acids, yielding a sugar, which was also laevorotatory and formed glucosazone. The product thus resembled the laevans synthesised by other sporing bacilli and plant pathogens.

*Bact. hyacinthi* only produced a trace of polysaccharide from sucrose (5 %), and a chemical examination was not possible. The organism failed to grow in 10 % sucrose solution.

*Chromobacter viscosum*. According to Harrison and Barlow [1905] this organism produces a slime from sucrose, lactose and galactose. We have confirmed these observations but have not been able to isolate the polysaccharide, owing to the small amount synthesised by the particular culture. Replacement of peptone by asparagine, glycine, alanine or leucine did not increase the yield, although the organism grew satisfactorily. There was no growth, however, with succinamide, malonamide, urea and methylurea as sources of nitrogen.

In the case of this organism, it has not been possible to stimulate enzyme-formation or to increase the amount of polysaccharide synthesised.

## Section II.

### *Polysaccharide formation by* Leuconostoc *species.*

Dextran formation by *Leuconostoc* was investigated by several earlier workers, especially by Liesenberg and Zopf [1892], Zettnow [1907] and Beijerinck [1912]. Tarr and Hibbert [1931], however, were the first to investigate accurately the conditions of formation of dextran by *Leuconostoc mesenteroides*, and these workers have provided an extensive bibliography. They found that the polysaccharide is synthesised by this organism in a peptone-phosphate medium from sucrose, but only in a very small amount from glucose and not at all from other carbohydrates. In the case of sucrose dextran production increased with rise in concentration up to 20 % and attained a maximum within a period of 10 days. Tarr and Hibbert were able to prepare large amounts of the dextran for chemical investigation.

We have continued these investigations with *L. mesenteroides*, the organism being grown at 30° in the same culture medium as described in the previous section. With two cultures the yield of "dextran" was very small indeed. We endeavoured to increase polysaccharide formation by replacing or supplementing the peptone by 0·1 % concentrations of various amino-acids (glycine, *d*-alanine, asparagine, *l*-leucine, glutamic acid). Only in the case of *l*-leucine was any difference noted, a perceptible increase in polysaccharide production occasionally being observed, but the improvement was not sufficiently marked for a large-scale synthesis. Subsequently we attempted to isolate active organisms from "leuconostoc slime". The inoculation of the 10 % sucrose culture medium with a particle of the slime rapidly caused opalescence after only 48 hours' incubation at 30°, and by precipitation with alcohol a dextrorotatory polysaccharide was obtained, yielding glucose after hydrolysis with acid. A culture was therefore plated out on sucrose-nutrient agar, and pure cultures were isolated and maintained on the same medium. None of these cultures, however, produced polysaccharide in a sucrose-peptone medium, notwithstanding the synthetic activity of the organisms in the original slime. Moreover, the capacity of the mixed slime organisms was lost on repeated subculture into fresh sucrose-peptone medium. We then found that Hucker and Pederson [1930; 1931] had stated that most *Leuconostoc* strains require an accessory growth substance, present in yeast or tomato extract, in addition to the ordinary peptone-nitrogen supply. Allison

and Hoover [1934] and Thorne and Walker [1934] also showed that certain nutritive factors present in yeast, molasses and egg albumin were essential for the growth of *Bact. radicicolum*, and our experience in this laboratory with this organism has fully confirmed their conclusions.

We therefore decided to investigate the influence of the presence of raw beet-sugar and molasses on polysaccharide synthesis by *Leuconostoc*, and samples for the purpose were kindly supplied by the West Midland Sugar Co., Ltd.

The cultivation of the foregoing organisms (isolated on the nutrient agar medium) in peptone-sucrose solutions, containing varying proportions of raw beet-sugar or molasses, however, did not lead to any improvement, as polysaccharide was still not produced. The organisms present in the original crude slime, on the other hand, maintained their dextran-producing capacity with repeated subcultivation in raw sugar media, although, as already pointed out, this was rapidly lost in pure sucrose cultures.

The technique of isolation of the organisms from the crude slime was, therefore, repeated, employing nutrient agar, containing 8% sucrose and 2% molasses, and the pure cultures were maintained on this medium (with the addition of calcium carbonate) throughout the experimental work. Certain of the colonies isolated during the process of plating out were now vigorously gum-producing and became surrounded by a highly viscous secretion. Four types of organism were finally selected and stock cultures kept on the molasses medium. The capacity of these cultures to synthesise dextran was tested in (*a*) 10% sucrose, (*b*) 6% sucrose+4% raw beet-sugar, (*c*) 8% sucrose+2% molasses, peptone being employed as source of nitrogen. The synthetic powers of two identified varieties of *Leuconostoc* kindly sent to us by Prof. A. J. Kluyver[1] were also compared in the same media.

The results are set out in Table II.

Table II.

| Organism | | Dextran-formation (6 days' incubation at 30°) (*a*) 10% Sucrose | (*b*) 4% Raw beet-sugar+6% sucrose | (*c*) 2% Molasses+8% sucrose |
|---|---|---|---|---|
| Organisms isolated from the slime | A | − | + + | + + |
| | B | − | − | − |
| | C | − | − | − |
| | D | + | + + | + + + |
| *Betacoccus arabinosaceus anhaemolyticus* (Kluyver) (*Leuconostoc mesenteroides*) | | + | + + | + + |
| *Betacoccus arabinosaceus haemolyticus* (Kluyver) (*Leuconostoc dextranicum*) | | + + | + + + | + + + |

− No dextran formation. + Slight. + + Moderate. + + + Maximum.

The results indicate that in the case of all synthesising cultures, dextran formation was greatly increased by the presence of raw beet-sugar or molasses. Only one organism (*B. arab. haemolyticus*) produced dextran actively in pure sucrose medium, and even this was stimulated by raw sugar; organism A, which produced great viscosity on molasses-agar, not only failed to synthesise dextran from pure sucrose but also failed to grow in this medium. Two organisms B and C did not possess any synthetic capacity in any of the three media employed.

*B. arab. haemolyticus* (*L. dextranicum*) produced great viscosity in sucrose solutions, with or without raw sugar or molasses, the viscosity being so marked

[1] These varieties of *Leuconostoc* are classified under the generic name *Betacoccus* (see Table II).

that the cultures could be safely inverted. In pure sucrose medium viscosity was well marked within 4 days; in the presence of raw sugar or molasses it was apparent in 2 days and sometimes in 24 hours. Notwithstanding this initial stimulation, it is important to note that after 10 days' incubation the yield of polysaccharide (by weight) was practically the same.

Proof that the polysaccharides synthesised by the foregoing organisms were of a dextran nature was afforded by the following evidence. The polysaccharides were precipitated by the addition of alcohol (equal volume) to the viscous cultures and purified by the method already described in Section I. The products from all these organisms were entirely soluble in hot water, giving dextrorotatory solutions. After prolonged hydrolysis with $N$ $H_2SO_4$ they yielded a sugar, which had the specific rotation (approximately) of glucose and formed glucosazone with phenylhydrazine.

On account of the readiness with which *L. dextranicum* synthesises a dextran from sucrose with the production of an easily-recognised viscosity, we have employed this organism in some further researches, with the object of throwing light on the two following problems:

(*a*) An explanation of the failure of this organism to produce dextran from glucose.

(*b*) The chemical nature and rôle of the so-called accessory growth factor present in raw beet-sugar and molasses.

(*a*) *Action on glucose.* The inability of this organism to synthesise dextran from 10 % glucose solution except in very small amount was first confirmed. It was observed that growth was poor and viscosity did not develop, and on adding alcohol to the glucose cultures only a turbidity or very small amount of precipitate was obtained. Identical results were obtained when very small amounts of beet-sugar or molasses were added to the glucose medium to stimulate the organism, and there was no improvement in yield by working at concentrations of glucose of 2 or 5 %.

This failure to produce dextran was not apparently due to the inhibitory effect of acid formation from the glucose, as the $p_H$ of glucose and sucrose cultures after a week's incubation was of very much the same order (about 4).

From these results it might be provisionally inferred that the dextran is synthesised from the fructofuranose section of the sucrose molecule.

We attempted to test this possibility by studying the effect of *L. dextranicum* on laevan synthesised by *B. pruni*. The laevan, however, was not attacked, and there was no evidence of dextran-formation.

An examination of the reducing sugars present in cultures of *L. dextranicum* in 10 % sucrose media was therefore next undertaken. It was found that after removal of the polysaccharide by alcohol the solutions were definitely laevorotatory, and from the optical activity and reducing power it was calculated that the reducing sugar present was mainly fructose, suggesting that the dextran had been built up from the glucose. Notwithstanding, a rôle might still have to be ascribed to fructofuranose in order to account for the dextran formation from sucrose only, and it seemed possible that, whilst the dextran is actually synthesised from the liberated glucose, yet the nascent fructofuranose might be essential in the constructive metabolism of the organism. However, in experiments in which small amounts of sucrose were added to glucose there was no utilisation of glucose for polysaccharide formation. On the contrary, the yield of dextran from sucrose was perceptibly diminished in the presence of excess of glucose.

At the present time no explanation can be offered to account for the slight growth and low synthesising activity of the organism in glucose media, compared with sucrose.

Experiments have also been carried out to ascertain whether there is evidence of the formation of a synthetical enzyme in a peptone medium containing 2% glucose and also 1% molasses. The organism was grown in this medium (10 ml.) for 3 days; at this stage toluene was added and also 10 ml. of 20% sucrose. Incubation was continued at 30° for a week, and the culture was then poured into three times its volume of alcohol. No precipitate was obtained, indicating that dextran had not been formed, and therefore an active synthesising enzyme had not been produced in the original culture.

The question as to whether *L. dextranicum* could utilise as a carbohydrate source the dextran it synthesised was next examined. The organism was subcultured into a peptone-phosphate medium, containing 1% dextran, and after 10 days the medium was tested for reducing sugar and change in $p_H$. No evidence of hydrolysis of the polysaccharide or production of acid was obtained, showing that the organism had not utilised the synthesised dextran as a reserve carbon supply under the conditions employed.

The results are analogous with those obtained by Anderson [1933] who found that the gum synthesised by *Rhizobium* was not utilised by the organism. In both cases polysaccharide synthesis appears to be an irreversible process.

(*b*) *The stimulating action of raw beet-sugar and molasses on polysaccharide formation.* In the first place experiments were carried out to ascertain the minimum amount of these substances essential to effect a definite stimulation. Varying amounts of raw beet-sugar or molasses were therefore added to sucrose-peptone cultures, the amount of pure sucrose being adjusted to maintain the sugar concentration at 10%. It was found that comparatively small amounts were adequate to accelerate the development of viscosity. 0·2% of either raw beet-sugar or molasses produced viscosity in 24 hours, whereas in this experiment the sucrose-peptone control culture required 96 hours. However, we also found that 0·5% of molasses could replace the peptone, viscosity being obtained in 24 hours. Therefore, the presence of available nitrogen in the molasses had to be considered.

When the peptone was replaced by 0·1% of any of the following nitrogen compounds: glycine, *d*-alanine, *l*-leucine, asparagine, glutamic acid (sodium salt), creatine, malonamide, succinamide, ammonium succinate, urea or methylurea, no growth or dextran formation took place in the absence of raw sugar or molasses, except on one occasion with *d*-alanine, when slight viscosity developed. The addition of small amounts of histidine, lysine or proline to supplement the foregoing nitrogen compounds or peptone was also without effect.

These results point to the conclusion that the essential substance present in beet-sugar and molasses cannot be identified with any of these amino-acids or amides.

The fact just mentioned that viscosity is produced in peptone-sucrose medium, but not in media in which the peptone is replaced by pure amino-acids and amides, suggested, however, that peptone contains a certain proportion of the substance present in raw sugar and molasses, essential for growth and dextran synthesis. The requisite structure could, of course, be either in the combined or free state in the peptone.

50 g. of peptone were therefore refluxed with 200 ml. of absolute alcohol for 24 hours, the mixture filtered and the filtrate concentrated *in vacuo* and finally made up with distilled water to 20 ml. It was sterilised before use. The

extracted peptone residue was again refluxed with alcohol, washed with alcohol and ether and dried in the steam-oven. Culture media containing 0·1 % of the original and the extracted peptone with 10 % sucrose and the usual salts were then prepared for comparison.

It was found (see Table III) that whilst the viscosity developed in the presence of the original peptone in 48 hours, with the extracted peptone it was delayed until 72 or 96 hours, and sometimes had not developed in 2 weeks. The addition of 0·05 or 0·1 ml. of the peptone extract to either original or extracted peptone on the other hand accelerated the development of viscosity.

The substitution of 0·05 ml. of the peptone extract for peptone as sole source of nitrogen also readily produced viscosity in 48 hours.

0·01 ml., however, contained a marginal amount of available nitrogen for the organism, as viscosity was postponed until the 10th day of incubation. The percentage of nitrogen in this extract was 0·375. It is interesting to record, however, that the addition of this limiting dose (0·01 ml.) of extract to culture media containing the extracted peptone did not accelerate the appearance of viscosity, and a similar addition to media containing 0·1 % asparagine, alanine, or ammonium succinate did not result in the development of viscosity any earlier than in the control experiment (10 days). Thus, when the amount of extract is so reduced, in order to restrict the available nitrogen, there is no evidence at all of any stimulation. We have been able, however, to demonstrate stimulation by using extracts of molasses under conditions where available nitrogen, if present, was inadequate. These extracts were prepared as follows. 100 g. of molasses were refluxed with alcohol for 6 hours, the mixture being shaken occasionally during this period. The clear alcoholic layer was poured off and the extraction of the molasses was repeated during 3 hours with 100 ml. of alcohol. The combined alcoholic extracts were evaporated under diminished pressure to a syrup which was dissolved in about 50 ml. of hot absolute alcohol and allowed to stand overnight at a temperature of 5–10°. This cooling caused the precipitation of sucrose and on pouring off the alcoholic solution and evaporating *in vacuo* a small amount of a brownish yellow residue was obtained. This residue was then extracted with 100 ml. of ether, the ether extract poured off and the ether-insoluble portion extracted with 100 ml. of alcohol-ether (50 : 50). The alcohol-ether extract was poured off and the insoluble portion was dissolved with 100 ml. of absolute alcohol. The three solutions thus obtained were evaporated *in vacuo* and each of the residues was taken up in water. The ether-soluble portion was made up to 10 ml., the ether-alcohol portion to 20 ml. and the alcohol-soluble residue to 20 ml. These solutions are referred to as extracts A, B and C respectively in Table III.

It was found that these extracts, unlike the peptone extracts, were deficient in available nitrogen, even 0·5 ml. per 10 ml. of culture medium being inadequate as sole source of nitrogen for sustaining growth and polysaccharide synthesis. On the other hand, very small amounts, *e.g.* 0·01 ml., accelerated in a remarkable degree the development of viscosity in the media containing peptone or extracted peptone.

There was evidence, moreover, that extract C (alcoholic) was more efficacious than the alcohol-ether extract B. The ether extract A contained the stimulating factor, but we do not know yet whether the content is less than in the case of B or C.

The results indicate that the stimulating action is not due to the nutritive effect of the presence of available nitrogen in the extracts, but is dependent on a distinct factor, which is being further investigated.

Table III. *Influence of peptone and molasses extracts on dextran formation in 10 % sucrose-phosphate. Total volume 10 ml.*

| Nitrogen compound 0·1 % | Peptone extract added ml. | Period required for development of viscosity (days) |
|---|---|---|
| Peptone | — | 2 |
| Extracted peptone | — | 4 |
| „ „ | 0·05 | 3 |
| „ „ | 0·01 | 4 |
| — | 0·05 | 2 |
| — | 0·01 | 10 |
| Glycine | — | — |
| *d*-Alanine | — | — |
| *l*-Leucine | — | — |
| Asparagine | — | — |
| Glutamic acid (Na salt) | — | — |
| Succinamide | — | — |
| Ammonium succinate | — | — |
| Creatine | — | — |
| Urea | — | — |
| Methylurea | — | — |
| Asparagine | 0·01 | 10 |
| *d*-Alanine | 0·01 | 10 |
| Ammonium succinate | 0·01 | 10 |

No viscosity in 10 days

| Nitrogen compound 0·1 % | Molasses extract added ml. | Period required for development of viscosity (days) |
|---|---|---|
| Peptone | — | 3 |
| „ | A 0·1 | 2 |
| „ | B 0·1 | 2 |
| „ | C 0·1 | 1 |
| „ | 0·05 | 1 |
| „ | 0·02 | 2 |
| „ | 0·01 | 2 |
| Extracted peptone | — | No viscosity in 10 days |
| „ | B 0·1 | 1 |
| „ | 0·05 | 2 |
| „ | 0·02 | 3 |
| „ | C 0·1 | 1 |
| „ | 0·05 | 1 |
| „ | 0·02 | 2 |
| „ | 0·01 | 3 |
| „ | B 0·1 | 3 |
| „ | 0·5 | 3 |
| , | C 0·1 | 1 |
| „ | 0·5 | 1 |

| Nitrogen compound 0·1 % | Molasses extract added ml. | Period required for development of viscosity (days) |
|---|---|---|
| — | A 0·5 | No viscosity in 10 days |
| — | B 0·5 | |
| — | C 0·5 | |
| Ammonium succinate | — | No viscosity in 10 days |
| „ „ | B 0·5 | |
| *d*-Alanine | — | |
| „ | C 0·5 | |
| Valine | — | |
| „ | C 0·1 | |
| *l*-Leucine | — | |
| „ | B 0·5 | |
| Glutamic acid (Na salt) | — | |
| „ „ | C 0·1 | |
| *l*-Histidine HCl | — | |
| „ | C 0·1 | |
| Asparagine | — | |
| „ | A 0·1 | |
| „ | B 0·1 | |
| „ | C 0·1 | |
| „ | C 0·5 | Slight viscosity in 7 days |

When the peptone was replaced by ammonium succinate and certain amino-acids (Table III) dextran formation did not take place, even in the presence of one of the stimulating extracts. In the case of asparagine with an excess of extract C, a slight viscosity developed after some delay (7 days). *Leuconostoc* is, therefore, much more specific in regard to its nitrogen requirements than the "laevan"-forming organisms, which synthesised the polysaccharide in certain amino-acid media as effectively as in the presence of peptone [Cooper and Preston, 1935].

*Large-scale preparation of the dextran synthesised by* Betacoccus arabinosaceus haemolyticus.

Equal volumes of sterile 20% sucrose solution and double strength peptone-salt medium (0·2% peptone) were distributed while hot into large Erlenmeyer flasks, the total volume of medium per flask being 800 ml. 5 ml. of sterile 50% molasses solution were added to all the flasks, which were then inoculated with a 48-hour culture of the organism in the same medium and incubated at 30° for 2 weeks. It was found advisable to avoid steaming the sugar and peptone solutions after admixture, as previous experience had shown that even in the presence of molasses the organism then occasionally failed to grow. The total volume of medium employed for the dextran synthesis was 5 litres.

After 2 weeks the contents of the majority of the flasks were highly viscous, and they were then poured into an equal volume of ethyl alcohol with vigorous stirring. The yield of crude dry polysaccharide was 121 g. (about 25% of the saccharose employed).

SUMMARY.

1. *Bacillus lactis* synthesises a polysaccharide from sucrose, but not from other carbohydrates. The product proved to be of the "fructosan" type, analogous to the "laevans" synthesised by other aerobic sporing bacilli and also plant-pathogens.

2. Dextran formation from sucrose by *Leuconostoc* species is greatly stimulated by the presence of raw beet-sugar and molasses.

3. There is evidence in the case of *L. dextranicum* (*Betacoccus arabinosaceus haemolyticus*) that the dextran is synthesised from the glucose portion of the sucrose molecule. Notwithstanding, even under varied conditions, the polysaccharide is only formed in very slight amount from free glucose.

4. Small amounts of raw beet-sugar, molasses or alcoholic extracts of peptone can supply the required available nitrogen for normal dextran synthesis, and can thus replace ordinary peptone in the culture media.

5. Alcoholic extracts of molasses, however, are deficient in available nitrogen, but minute proportions greatly accelerate growth and dextran formation in either peptone or alcohol-extracted peptone media. The stimulating action is thus dependent on some other factor than nitrogen supply.

6. Polysaccharide formation does not take place in media in which peptone has been replaced by certain amino-acids or ammonium succinate (Table III).

7. A method is described for the large-scale preparation of the dextran synthesised by *L. dextranicum*.

This investigation has been carried out during the tenure of a Leverhulme Research Fellowship by one of us (E. A. C.).

The authors desire to express their thanks to Prof. W. N. Haworth and Dr E. L. Hirst for their interest and encouragement; and to Dr R. St John Brooks, Dr G. J. Hucker, and Prof. A. J. Kluyver for kindly supplying the micro-organisms used in this research.

## REFERENCES.

Allison and Hoover (1934). *J. Bact.* **27**, 561.
Anderson (1933). *Iowa Agric. Exp. Sta. Res. Bull.* **158**, 27.
Beijerinck (1912). *Folia Mikrobiol.* **1**, 377.
Cooper and Preston (1935). *Biochem. J.* **29**, 2267.
Harrison and Barlow (1905). *Cent. Bakt.* II, **15**, 557.
Hucker and Pederson (1930). *N.Y. State Agric. Exp. St. Tech. Bull.* No. 167.
—— —— (1931). *Cent. Bakt.* II, **85**, 65.
Liesenberg and Zopf (1892). *Cent. Bakt.* **12**, 659.
Tarr and Hibbert (1931). *Can. J. Research*, **5**, 414.
Thorne and Walker (1934). *Proc. Iowa Acad. Sci.* **41**, 63.
Zettnow (1907). *Z. Hyg.* **57**, 154.